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Enhancement of the antileukemic activity of 5-aza-2′-deoxycytidine by cyclopentenyl cytosine in HL-60 leukemic cells

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We have investigated the capacity of cyclopentenyl cytosine (CPE-C), a potent inhibitor of CTP synthetase, to modulate the antineoplastic activity of 5-aza-2′-deoxycytidine (DAC) on HL-60 myeloid leukemic cells. The combination of CPE-C and DAC produced an additive effect on the growth inhibition of the cells following a treatment of 48–96 h. Cytotoxicity experiments measured by the cloning of cells in soft agar following 24 and 48 h exposures produced a more than additive effect when the drugs were used in combination. Evaluation of the effect of CPE-C and DAC on the induction of differentiation of HL-60 cells following a 48 h treatment revealed that the combination of the drugs produced a more than additive effect than when the drugs were used alone. Measurement of the intracellular pool of deoxycytidine triphosphate (dCTP) showed that a 6 h exposure to 0.05 and 0.1 μM of CPE-C reduced the pool by 60 and 88%, respectively. The decrease in the dCTP pool was correlated with a higher incorporation of radioactive DAC into DNA. The deamination of CPE-C to cyclopentenyl uridine by cytidine deaminase was investigated with the purified enzyme from human placenta. We report here that CPE-C is a very poor substrate for cytidine deaminase as compared with cytidine. These studies suggest that CPE-C could be used as a biochemical modulator to increase the antileukemic action of DAC.

Key words: 5-Aza-2′-deoxycytidine, cyclopentenyl cytosine, HL-60, leukemic cells.

Introduction

The cytidine analog cyclopentenyl cytosine (CPE-C) is a new and very interesting cytotoxic agent in which the furan ring of the ribose sugar has been replaced by a cyclopentenyl moiety.[1] CPE-C has been reported to have antineoplastic activity *in vivo* against several types of human tumor xenografts and murine leukemic cells in mice.[2,3] Cells resistant to the antimetabolite cytosine arabinoside (ara-C) due to a deficiency in deoxycytidine kinase (dCK) are still sensitive to CPE-C.[3,4]

In order to be active CPE-C must be phosphorylated to its triphosphate form (CPE-CTP) through the uridine/cytidine salvage pathway.[5] CPE-CTP is a potent inhibitor of cytidine triphosphate synthetase (CTP synthetase) producing a reduction of the CTP and deoxycytidine triphosphate (dCTP) pools, and resulting in an inhibition of DNA and RNA synthesis.[5,6] CPE-C has also shown the capacity to induce the differentiation of HL-60 leukemic cells.[7] Pharmacokinetic studies in rodents and dogs indicate that renal excretion is the primary route of excretion of CPE-C,[8] but in monkeys deamination of CPE-C to CPE-U is the primary route of elimination.[9] Cellular studies on CPE-C in Molt-4 lymphoblastic cell line demonstrated the capacity of this cell line to deaminate CPE-C.[10]

5-Aza-2′-deoxycytidine (DAC) is an experimental deoxycytidine analog that shows more potent antileukemic activity in animal models than ara-C.[11,12] In phase I studies in man DAC was demonstrated to be an active antileukemic agent.[13,14] DAC must undergo phosphorylation in order to be an active antimetabolite. The phosphorylation is catalyzed through the dCK metabolic pathway. DAC is also subject to degradation by cytidine deaminase (CDA) to 5-aza-2′deoxyuridine (DAU) resulting in a loss of activity.[15] The incorporation of 5-aza-2′-deoxycytidine triphosphate (DACTP) into DNA produces an inhibition in DNA methylation resulting in the activation of specific genes and induction of cell differentiation.[16–19].

CPE-C has been reported to modulate the cytotoxicity of arabinosyl cytosine analogs.[20] In this report we wanted to investigate the capacity of CPE-C to enhance the antiproliferative action of DAC in HL-60 myeloid leukemic cells.

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Materials and methods

Materials

DAC was obtained from Mack Co. (Jllertissen, Germany), dissolved in 0.45% NaCl containing 10 mM sodium phosphate pH 6.8 and stored at −70°C. [6-^{3}H]DAC was obtained from Moravek Biochemicals Inc. (Brea, CA). CPE-C was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (NIH), Bethesda, MD. Nitroblue tetrazolium (NBT) and phorbol myristate acetate (PMA) were obtained from Sigma (St Louis, MO).

Cell culture

Human HL-60 leukemic cell line was obtained from Dr R Gallo, National Cancer Institute. The cells were maintained in suspension culture in minimal essential medium (MEM) containing non-essential amino acids (Gibco, Grand Island, NY) and 10% heat-inactivated fetal calf serum (Flow Laboratories, Mississauga, Ontario, Canada). The doubling time of the cell line was between 20 and 22 h.

Inhibition of cell growth and colony assay

For the growth inhibition studies HL-60 leukemic cells in log growth phase were placed at a density of 25 000 cells/ml in 5 ml of medium containing indicated amount of drug. At 48, 72 and 96 h the cells were counted with a model ZH Coulter Counter. The proliferative viability of the leukemic cells after exposure to DAC and/or CPE-C was determined by cloning in soft agar. At termination of drug exposure the cells were centrifuged and suspended in drug-free medium. An aliquot of 150–200 cells was placed in 2 ml of 0.15% soft agar medium containing 16% serum. After incubation for 14–16 days at 37°C in a 5% CO_2 incubator, the number of colonies (>500 cells) was counted. The cloning efficiency of the control cells was in the range of 40–60%.

Effect of CPE-C on the pool of dCTP in HL-60 cells

The leukemic cells at a density of 10^5 cells/ml in 50 ml were treated for 6 h with different concentrations of CPE-C in MEM medium containing 5% dialyzed serum. After the end of drug exposure the cells were centrifuged, and treated to perchloric acid and trioctylamine/freon to allow the isolation of the intracellular nucleotides. The concentration of dCTP in the extracts was determined by the method developed by Shermann and Fyfe[21] using a synthetic matrix of oligonucleotides (Research Genetics, Huntsville, AL).

Incorporation of [^{3}H]DAC into DNA of leukemic cells

Cells at 2×10^5/ml in 2 ml of MEM medium containing 5% dialyzed serum were incubated with 0.05 μM of [^{3}H]DAC and various concentrations of CPE-C for 6 h at 37°C. At the end of the incubation the cells were placed on Whatman GF/C glass fiber filters (2.4 cm diameter) that were washed previously with 0.9% NaCl. The filters were then washed with 0.9% NaCl, cold 5% trichloroacetic acid, ethanol, dried and placed in scintillation fluid (Omnifluor, Dupont) for the determination of radioactivity incorporated into DNA.

Evaluation of differentiation of HL-60 leukemic cells

Cells were analyzed for their ability to reduce NBT to formazin as described by DeChatelet *et al.*[22] Briefly, 2×10^5 cells/ml in 5 ml of MEM medium containing 5% dialyzed serum were incubated for 48 h with different concentrations of DAC and/or CPE-C. At the end of the incubation, the cells were centrifuged and incubated for another 48 h in fresh medium containing no drugs. For the differentiation evaluation, 2×10^5 cells were placed in 1 ml of MEM medium and mixed with 1 ml of NBT (1.0 mg/ml). After the addition of PMA (0.25 μg/ml), the cells were placed in a shaker bath at 37°C for 40 min and the fraction of NBT postive cells was evaluated microscopically using a hemocytometer.

Cytidine deaminase assay

Purification of the enzyme is described elsewhere.[23] The specific activity of the purified CDA was 11 000 units/mg protein; 1 unit of enzymatic activity being defined as the amount of enzyme that catalyzes the deamination of 1 nmol cytidine/min at 37°C. CDA activity was determined by spectrophotometric assay. The reaction mixture (0.2 ml) contained

20 mM $KHPO_4$, pH 7.5, 100 mM KCl, the indicated concentration of substrate and about 0.15 μg (1.5 units) of the purified CDA. The mixture was placed in a 10 mm light-path cuvette and incubated at 37°C in a Gilford 260 spectrophotometer. The rate of deamination was determined by the decrease in absorbance at 286 nm. A ΔE_M value of 3000 at 286 nm was used to calculate the amount of substrate deaminated. In this assay the daily deviation was less than 10%.

Results

The effect of DAC and CPE-C alone or in combination on the growth of HL-60 leukemic cells is shown in Table 1. Both DAC and CPE-C were active inhibitors of the growth of HL-60 cells. The combination of DAC and CPE-C showed a more than additive effect on the growth of HL-60 leukemic cells. After an incubation of 72 h with DAC 0.05 μM alone and CPE-C 0.02 μM alone produced 24.7 and 17.8% inhibition, respectively, of the growth of HL-60 cells. These drugs in combination produced growth inhibition of 56.7%, a more than additive effect. For a 96 h incubation with the same concentrations there was a 31% inhibition of growth for DAC and CPE-C alone whereas the combination gave a 60% inhibition suggesting an additive effect.

The cytotoxic action of DAC and CPE-C alone or in combination on HL-60 leukemic cells was investigated using a colony assay (Table 2). The combination of the drugs produced a more than additive effect than when the drugs were used alone. A 24 h incubation of HL-60 leukemic cell line to 0.05 μM of DAC or 0.02 μM of CPE-C produced 24.1 and 9.8% relative cell kill to the cells, respectively. When the same concentrations of the drugs were used in combination there was a 42.1% relative cell kill which demonstrates a more than additive effect on the clonogenicity of the cells as evaluated by the method of Valeriote and Lin.[24] The same phenomenon was observed for a 48 h treatment. DAC 0.05 μM and CPE-C 0.04 μM used alone gave cytotoxicity values of 33.8 and 20.5%, respectively, and when the agents were used in combination at the same concentrations there was a 82.2% cytotoxicity.

Table 2. Effect of DAC and/or CPR-C on colony formation in HL-60 leukemic cells

Drug	Concentation (μ)	Relative cell kill (%)	
		24 h	48 h
DAC	0.05	24.1 ± 1.1[a]	33.8 ± 1.0
CPE-C	0.02	9.8 ± 0.4	16.1 ± 4.5
CPE-C	0.04	15.0 ± 2.8	20.5 ± 3.8
DAC + CPE-C	0.05 + 0.02	42.1 ± 2.4	52.6 ± 1.0
DAC + CPE-C	0.05 + 0.04	60.8 ± 4.0	82.2 ± 2.3

The cells were exposed with the indicated amount of drugs for 24 or 48 h and their proliferative viability was evaluated by cloning in soft agar.
[a] Mean ± SE (n = 3).

The effect of CPE-C on the intracellular pool of dCTP in HL-60 myeloid leukemic cells is shown in Table 3. An increase in CPE-C concentration resulted in a proportional decrease in the dCTP pool in this cell line. At a concentration of 0.01 μM the effect of CPE-C was not significantly different from the control value (9.5 ± 0.8 and 10.3 ± 0.3 μM, respectively). The effect of CPE-C at higher concentrations showed a significant decrease in the dCTP concentration in the cells. When the cells were exposed to 0.05 and 0.1 μM the reduction of the pool of dCTP reached 60 and 80%, respectively. There was a >99% reduction in the pool of dCTP when we used a concentration of CPE-C of 1 μM.

Since CPE-C can decrease the intracellular pool of dCTP, we investigated the effect of CPE-C to modulate the incorporation of DAC into the DNA of HL-60 leukemic cells (Figure 1). There was a correlation between the amount of DAC incorporated into DNA of the cells and the concentration of CPE-C. A con-

Table 1. Growth inhibiation of HL-60 myeloid leukemic cells by DAC and/or CPE-C

Drug	Concentration (μM)	Growth inhibition (%)		
		48 h	72 h	96 h
DAC	0.05	21.5 ± 1.5[a]	24.7 ± 1.5	31.0 ± 1.0
CPE-C	0.01	3.9 ± 1.6	6.7 ± 2.9	7.7 ± 1.2
CPE-C	0.02	10.9 ± 2.8	17.8 ± 3.5	31.4 ± 1.0
DAC + CPE-C	0.05 + 0.01	30.1 ± 0.9	33.6 ± 1.5	34.1 ± 0.4
DAC + CPE-C	0.05 + 0.02	37.2 ± 2.4	56.7 ± 1.5	60.4 ± 1.3

HI-60 leukemic cells at a density of 25 000 cells/ml were treated with the indicated amount of drugs and the cells were counted 48, 72 and 96 h after the start of treatment to evaluate the degree of inhibition caused by the drugs.
[a] Mean ± SE (n = 3).

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